A MEMBER OF THE MYCOBIOME MODULATES A HOST METABOLITE TO INCREASE INTESTINAL PERMEABILITY AND DISEASE

by

Tyson Richard Chiaro

A thesis submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Master of Science

in

Laboratory Medicine and Biomedical Science

Department of Pathology

The University of Utah

May 2015

The University of Utah Graduate School

STATEMENT OF THESIS APPROVAL

The following faculty members served as the supervisory committee chair and members for the thesis of **Tyson Richard Chiaro**.

Dates at right indicate the members' approval of the thesis.

June Round, Chair	March 12, 2015 Date Approved
Matthew Mulvey, Member	March 12, 2015 Date Approved
Janis Weis, Member	March 12, 2015 Date Approved

The thesis has also been approved by **Peter Jensen** Chair of the Department/School/College of **Pathology** and by David B. Kieda, Dean of The Graduate School.

ABSTRACT

The human body is a fascinating arrangement of cellular organization necessary to support life; it is also home to trillions of microorganisms. The largest concentration of these commensal microbes exists within the gastrointestinal tract (GI) where bacteria dominate the landscape. Accordingly, the vast majority of research performed thus far has been to elucidate the role of bacteria; however, commensal fungal species are known to colonize every mucosal surface, including the GI of humans. Moreover, humoral responses to fungal cell walls have been shown to be a specific diagnostic marker for Crohn's disease and not ulcerative colitis. However, the role of yeast within the GI both metabolically and immunologically remains largely unexplored. For this reason, we set out to investigate how a known commensal yeast species, *Saccharomyces cerevisiae*, and a newly described yeast species in the context of human disease, *Rhodotorula aurantiaca*, influence immune system development and modulate disease. Also, given the role of environmental fungi in nutrient cycling on the planet, we were interested in how these two species may impact the metabolism of the host. To address these questions, we used two common mouse models of colitis. In addition, we performed a microarray on colonic RNA from germfree mice mono-colonized with our two yeast species and LC/MS on the serum of these animals. Here we report that mono-colonization with *S. cerevisiae* but not *R. aurantiaca* results in a significant increase in products of the purine degradation pathway, including uric acid. Surprisingly, yeast did not induce a robust inflammatory response; however, we observed multiple pathways associated with both digestion and epithelial barrier integrity. We demonstrate that animals treated with *S. cerevisiae* during DSS colitis have worse colonic histology, increased serum uric acid levels, and intestinal permeability. Furthermore, we determined that supplementing uric acid alone during DSS colitis recapitulates these effects. We postulate that *S. cerevisiae* alters the colonic histology and intestinal permeability through

either direct lytic damage of cells or secretion of fungal metabolites that are in turn taken up by enterocytes and shunted into the purine salvage pathway, resulting in conversion to uric acid and reabsorption into the bloodstream.

This master's thesis is dedicated to my mother, a cancer-survivor, for her continual strength, support, and love.

Look deep into nature and then you will understand everything better.

- Albert Einstein

TABLE OF CONTENTS

LIST OF FIGURES

PROLOGUE

In recent years, the notion of intestinal health has tantalized American households with vigorous marketing campaigns claiming relief from an array of intestinal ailments. Ilya Mechnikov first proposed the idea of beneficial intestinal bacteria in his 1907 book, “The Prolongation of Life.” And while the concept of beneficial microorganisms is far from a new one, a century later, modern-day technologies are beginning to unravel the enormous complexity and vast consortium of microorganisms that comprise the mammalian gastrointestinal tract, collectively referred to as the *microbiota.*

INTRODUCTION

The Microbiota and Their Role in Host Physiology

The Human gastrointestinal tract (GI) is home to all three domains of life, including a substantial virobiome.[1-3] Enumerating the amount of microorganisms in this ecosphere can be a daunting task. While controversial, it is often noted that the GI contains ten times the amount of bacterial cells as cells in the human body[4,5]. However, these claims are often not cited and others have disputed this claim altogether.[6,7] Regardless of the exact number of microorganisms residing in the GI, it is clear that the number of bacteria, archaea, fungi, and viruses living in and on the human body is immense.[1,6,7] Bacteria, however, are the dominant kingdom in the GI and for this reason have been studied the most extensively. Prokaryotes within the gut perform a myriad of useful functions that aid the body in digestion, immune regulation, and even behavior.[8,9] Specific organisms within the gut contain enzymes that human cells lack; these enzymes facilitate the utilization of otherwise undigested carbohydrates.[10] Bacteria convert complex sugars such as, starches, fiber, and oligosaccharides into short chain fatty acids, such as acetic acid, propionic acid, and butyric acid, via a process known as saccharolytic fermentation.[11] These acids provide energy-rich carbon sources for epithelial cells and assist the body in the absorption of dietary minerals like calcium, magnesium, and iron.[12] These metabolites have also been shown to promote colonic homeostasis by binding to T regulatory cells within the gut and promote a regulatory phenotype.[13] Multiple studies have demonstrated that commensal bacteria can promote both tolerogenic and inflammatory responses; thus, the need for an appropriate microbiota is essential for host health. Accordingly, the immune system in the GI has a colossal task of discriminating between commensal organisms that provide benefit and potential pathogens.[11,14] Fungi, however, have been largely ignored in the context of their potential benefit to the host. Nonetheless, both the skin and the GI are known to house

several hundred species of yeast with *Malassezia* and *Candida* spp being the most prevalent, respectively.[15] Recently, several studies have emerged characterizing the mycobiome and what impact fungi have on host physiology. However, the contribution both immunologically and metabolically that yeast provide the host remains largely unknown.

It's a Fungal World

Fungi represent a diverse group of eukaryotic organisms that include yeasts, molds, and mushrooms. Fungi have, arguably, had a more positive influence on human health than any other microorganism. The budding yeast *Saccharomyces cerevisiae*, more commonly known as bakers yeast, has been used for centuries in rising bread and beer brewing. Alexander Flemming's discovery that the mold, *Penicillium notatum,* inhibited colonies of staphylococci in 1928 would revolutionize the medical community, lead to a Nobel Prize, and was quite possibly the most influential discovery of the twentieth century. Psychotropic compounds, such as psilocybin, found in some species of mushrooms have been used in religious ceremonies for millennia and have been reported to induce highly spiritual and positive emotions in a blinded study using psilocybin naïve volunteers.[16] Lastly, humans have suffered the ills of bacteria for millennia: Bubonic plague, epidemics of Cholera, scarlet fever, diphtheria, typhoid fever; sexually transmitted diseases such as syphilis, gonorrhea, and chlamydia, killed millions before the advent of antibiotics and even today remain problematic in the age of antibiotic resistance. While several species of fungi have evolved to be infectious in plants, comparatively speaking, few fungi are true pathogens in human hosts. Undeniably, the AIDS epidemic of the 1980s created the field of medical mycology. Along with advances in transplant technology, chemotherapy, and broad-spectrum antibiotics, the significance of fungal opportunists has become far more appreciated in the past 30 years. Fungal species such as *Candidia albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Aspergillus flavus, A. fumigatus*, *Pneumocystis jirovecil*, and the black mold *Stachybotrys chartarum* are common opportunistic pathogens seen in immunocompromised patients. Unlike bacterial or viral infections, which can be spread from person to person, fungal infections are not and are only acquired from the environment or other exogenous sources, as was the case in 2012 when injectable steroids became contaminated

with the fungus *Exserohilum rostratum.*[17] The ubiquitous nature of fungi in the environment and their ability to be manipulated for human use, along with their consistent presence in human hosts and the lack of true pathogens, suggests that the immune system has evolved sufficient control and regulatory mechanisms for fungi.

Functional Crosstalk Between the Host and the Microbiota

The mammalian immune system is classically known for its ability to recognize and clear pathogens from the body. However, the vast majority of microorganisms surveyed by the immune system are symbionts that live on and in mammalian hosts. In order to permit a complex microbial community to live and thrive within the body, the immune system has evolved several elegant strategies to compartmentalize these microbes in order to carry out their functions, while not mounting metabolically taxing immuno-inflammatory reactions. Both the innate and adaptive arms of immunity participate in the modulation of host immunity and are crucial for maintaining homeostasis in the GI.[18,19]

Pattern recognition receptors (PRR) such as toll-like receptors (TLR) and C-type lectins, located on phagocytic as well as intestinal epithelial cells recognize microbe-associated molecular patterns (MAMPS). Certain PRR have proven to be important for detection of fungi via the binding of sugar moieties in the cell walls of fungi. β-Glucan, for example, comprises at least 40% of the dry weight of *C. albicans;* accordingly, mammals have evolved two receptors that recognize β-glucan: Dectin-1 and complement receptor 3 (CR3).[20] Dectin-1 is a C-type lectin originally found on dendritic cells able to provide T cells with co-stimulatory signals; however, subsequent research has shown that Dectin-1 is primarily located on myeloid phagocytes where it acts to initiate phagocytosis.[20] Ligation of Dectin-1 by zymosan has been demonstrated to phosphorylate an ITAM-like motif on Dectin-1 and lead to IL-2 and IL-10 production.[21] The role of CR3 in β-glucan recognition is less defined, though it has been shown that CR3 signals through Src/Syk mechanisms and is important for ROS production in neutrophils in response to β-glucans.[20] Mannan, like β-glucan, is another rich carbohydrate in the cell walls of fungi. Macrophage mannose receptor (MR) is widely expressed by both macrophages and dendritic

cells and has been show to associate with TLR-2 during recognition of *Saccharomyces*, *Candida*, and *Pneumocystis*.[19,20] Blockade of the MR has been shown to reduce inflammatory cytokines; however, little is known regarding mannose receptor signaling. Dectin-2, a C-type lectin found on macrophages, has been shown to bind to fungal hyphae, by associating with the Fc-R γ-chain leading to TNFα and IL-1ra production.[21] While dimorphic yeasts such as *C. albicans* contain mannan in their cell walls and hyphal projections, Dectin-2 preferentially binds the latter.[21] Consequently, the disparity in cytokine production between Dectin-1 and Dectin-2 has been proposed to correlate with the clinical outcomes, yeast-dominant infections promoting a dampening of the immune response and hyphae-dominant infections resulting in inflammation.[21] TLR-2 and 4 are key receptors in recognizing multiple pathogenic fungi and are highly expressed on phagocytic cells and enterocytes.[20,22] However, how these particular receptors modulate fungal communities within the GI, given they are likely fully activated by bacteria, is not completely understood. Thus, it is clear that antigen-presenting cells can activate tolerogenic or immunogenic responses, depending on the antigen presented.[23] Furthermore, IgA secreted from plasma cells represents an example of adaptive immunity in the gut, as IgA has been shown to coat luminal bacteria, although how a humoral response may control fungal populations within the lumen has yet to be elucidated.[18]

Under homeostatic conditions, these processes allow the host and its microbial community to coexist peacefully; however, when these mechanisms break down, an aberrant immune response ensues and chronic inflammation develops. Delineating how tolerance to intestinal microbiota is maintained and subsequently lost, resulting in chronic inflammation, is of particular interest to researchers and clinicians studying and treating chronic inflammatory disorders of the bowel.

IBD: A Microbiota-linked Disease

Inflammatory bowel disease (IBD) is an idiopathic disease comprised of two types of chronic intestinal disorders: Crohn's disease (CD) and ulcerative colitis (UC). IBD affects approximately 1.4 million Americans, with most patients being diagnosed between the ages 15

to 30 years of age.[24] CD is a disease most commonly associated with the ileum of the colon; however, it can affect any region of the intestine and is often discontinuous in its presentation.[25] UC primarily affects the rectum but can affect any part of the colon or the whole colon in an uninterrupted pattern.[25] Advances in sequencing technology have revealed consistent taxonomic differences amongst mucosally–associated microbes in CD patients.[26] Genome-wide association studies have identified several risk alleles associated with the development of IBD; thus, both the genotype of the host and the environment can contribute to disease.[14,26] As population dynamics shift in the GI, either due to genetic deficiencies or exogenous factors such as diet or antibiotics, a bloom of organisms that promote inflammation can occur.[26,27] This imbalance of luminal microbiota is collectively referred to as "dysbiosis". One of the most distinguishing clinical markers for CD is the presence of antibodies to epitopes within fungal cell walls, specifically phosphopeptidomannan[28]. This humoral response has colloquially been termed: anti-*saccharomyces cerevisiae* antibodies (ASCA). However, these complex sugars with chitin backbones make up the molecular matrix in multiple phyla of yeast, including *Candida* and *Saccharomyces spp*, which are both known to colonize the mammalian GI and have been shown to contain the epitopes necessary to bind ASCA[2,29]. ASCA, therefore, are not specific to *Saccharomyces spp* and are most likely induced by *Candida spp* given *Candida's* prevalence in the GI[2,15]. Moreover, patients suffering from CD are heavily colonized with *C. albicans*; thus, fungal overgrowth in the presence of inflammation leads to increased recognition by professional antigen presenting cells and a secondary humoral response.[15,30] This suggests that fungi play a role in the prognosis of IBD.

Colonization: The Major Players

The vast majority of research regarding dysbiosis of the intestinal microbiota, including metabolic and immune functionality, has focused primarily upon its prokaryotic members. Their sheer numbers and their potential to be pathogenic qualify them as the predominant kingdom in a biome that contributes greatly to host health. Nevertheless, *Candida* spp. including *albicans, galbrata,* and *tropicalis* have been shown to dominate the fungal community in multiple mucosal

sites, including the oral cavity, vagina, and colon of healthy individuals.[2,31] The oral cavity has even been shown to consist of several genera traditionally thought to be pathogenic, including *Aspergillius, Fusarium,* and *Cryptococcus* spp.[32] It has been known for years that Candidiasis can result from antibiotic therapy leading to a disruption of microbial communities and the overgrowth of *Candida spp.*[15] However, what influence fungal communities have on bacterial populations and vice versa is just now beginning to be appreciated. How do fungal communities influence chronic inflammation and how is the dysbiosis of the mycobiome related to disease? These are questions investigators have recently started to address; however, the lack of standardized protocols, sequencing databases, and bioinformatics approaches will have to be resolved in order to overcome the variability seen in different studies thus far.[15]

A Novel Yeast Identified in Crohn's Disease Patients

In an effort to characterize the mycobiome in a cohort of CD patients, Ottman et al. evaluated the feces and mucosal milieu for differences in fungal communities. A significant difference was observed between mucosally associated and fecal fungi. In addition, fungal richness was appreciably higher in CD patients. Interestingly, the red pigmented, environmental Basidiomycete, *Rhodotorula aurantiaca,* was present in colonic mucosa of 19% of patients with UC and 13% of CD patients.[31] By contrast, *S. cerevisiae* was present in the stool of these patients, but was not identified in the mucosa. *R. aurantiaca* is an ubiquitous yeast that has been isolated from polar ice caps, soil, air, fruit juices, and indwelling catheters.[33] Although not usually pathogenic, *Rhodotorula* species have been reported to cause mycosis in immunocompromised patients and have been acknowledged in the GI in other studies.[34] In the mid-1980s, cases of fungemia due to *Rhodotorula spp* increased and for the first time, it was recognized as an opportunistic pathogen.[33] Eight species are associated with the genus *Rhodotorula*; however, only *R. mucilaginosa, R. glutinis, and R. minuta* have been reported to cause disease in humans.[33]

Thesis: How Does the Mycobiome Affect Host Health?

Due to the differential location of *R. aurantiaca* and *S. cerevisiae* in a study of IBD patients and the known fungal component for the diagnosis of CD, we set out to explore the immunologic and metabolic contribution of *R. aurantiaca* and *S. cerevisiae* in the GI. We orally gavaged each yeast while employing two different models of chemically induced colitis to study how each species modulates inflammation. We hypothesized that due to the appearance of *R. aurantiaca* in the colonic mucosa of CD patients, this would lead to an inflammatory state and exacerbate disease. Previous research using Balb/c gnotobiotic mice has shown that upon colonization with *C. albicans*, in the absence of microbiota and functional T cells, animals were resistant to extensive mucocutaneous and systemic candidiasis.[35] Therefore, given the work done previously with yeast and germfree mice and the observation of *S. cerevisiae* and *R. aurantiaca* in IBD patients, we wanted to know how yeast influence host physiology from a transcriptional perspective. Therefore, we mono-colonized germfree mice and took fecal samples to evaluate the metabolic capacity individual yeast species confer upon their hosts. As the mycobiome represent a minute portion of intestinal contents, we suspected that yeast would not influence the metabolic capacity of the germfree mouse significantly; however, we were interested if any metabolites in particular could be predicted to influence microbial ecology or affect the intestinal mucosa. Accordingly, this masters thesis was to study how fungi, specifically yeast, modulate innate and adaptive immune responses, influence the metabolic activity, and effect microbial ecology within the murine gastrointestinal tract.

RESULTS

S. cerevisiae Exacerbates Pathology in Two Experimental Models of Colitis

Chronic inflammatory disorders such as Crohn's disease result when tolerogenic mechanisms of gut-associated lymphoid tissue (GALT) breakdown, leading to aberrant immune responses to innocuous microbes.[36] This loss of tolerance leads to decreases in species richness and variation among several taxa comprising the mucosal-associated microbiome.[26] Given the humoral response seen in CD patients, and the presence of *R. aurantiaca* and *S. cerevisiae* being highly represented in the colonic mucosa and stool, respectively, of CD patients; we set out to explore how these yeast influence experimental colitis. Using a T-cell-driven model of colitis: 2, 4, 6–trinitrobenzene sulfonic acid (TNBS), C57BL/6 mice were orally gavaged with 1.0 x 10^6 CFU of *S. cerevisiae* or *R. aurantiaca* daily for 7 days, TNBS colitis was induced and followed for 5 days. Disease severity was assessed by percent weight loss and histology of the colon. Percent weight loss between treatment groups and control never reached significance over three experiments; however, there is a consistent trend of *R. aurantiaca* recovering sooner and more rapidly than *S. cerevisiae,* which is demonstrated when the average weight loss of each cohort over three independent experiments are compared by a students t-test (Figure 1. A, B). Inflammation of the colon was examined by histology; H&E-stained day 5 colonic sections of TNBS treated mice, orally gavaged with *S. cerevisiae,* showed a high degree of leukocyte and lymphocyte infiltration into the mucosa, alteration of epithelial structure, and near complete crypt loss when compared to untreated controls and mice orally gavaged with *R. aurantiaca* (Figure 1. C). Histological scores were generated from blind scoring of H&E-stained colonic tissues (Figure 1. D) In an attempt to explain the altered histology seen in the *S. cerevisiae* cohort, MLNs were processed and stained for the same effector T-cell subsets that

were examined previously. Surprisingly, we saw no differences in T-cell subsets, leaving the mechanism responsible for the altered histology open to further investigation.

We next used a model of dextran sodium sulfate (DSS) to explore what effects the exogenous addition of yeast has during an experimental model of colitis when barrier function of the colonic mucosa is disrupted. C57BL/6 mice were orally gavaged with $1.0x10^6$ CFU of *S. cerevisiae* or *R. aurantiaca* daily for 7 days; 2.5% (w/v) DSS was then added to the drinking water for 8 days. Mice continued to receive daily gavage of live yeast until sacrifice. Here we report that animals treated with *S. cerevisiae* lost more weight by day 7 during DSS colitis compared to controls and animals treated with *R. aurantiaca* (Figure 2. A, B). We also demonstrate that animals treated with *S. cerevisiae* show altered histology compared to DSS controls and those treated with *R. aurantiaca,* recapitulating the effect seen during TNBS colitis (Figure 2. C, D). Interestingly, MLNs from animals treated with *R. aurantiaca* showed a significant decrease in the amount of Th17 cells and IL-17 production when compared to DSS controls and animals treated with *S. cerevisiae* (Figure 3. A-D). qRT-PCR performed on RNA collected from colons of treated animals confirmed a significant decrease in *IL-17F* transcripts from animals treated with *R. aurantiaca* during DSS colitis (Figure 3. E).

These experiments provide evidence that certain yeast exacerbate disease pathology as can be seen when animals are treated with *S. cerevisiae* in two separate models of experimental colitis. Inflammatory T-cell subsets are known to play a role in the pathogenesis of IBD and experimental colitis; therefore, in the absence of clear differences, these cell types implies that yeast are not inducing a robust inflammatory response, and in the case of *R. aurantiaca,* are even suppressing certain inflammatory T-cell subsets.

Microarray Analysis of Germfree Animals Mono-colonized with Commensal Fungi

Immune system development depends on the presence of an intact microbiota. Indeed, germfree mice have deficiencies in multiple aspects of immune system development that can be complemented by providing a complex microbial community. Germfree mice provide a tool to

understand how a single microbe can dictate immune system development. To understand how *S. cerevisiae* resulted in worse colonic histology in two different models of colitis, as well as how yeast, in general, might skew innate and adaptive immune responses in the gastrointestinal tract, we took an unbiased approach and looked at gene expression profiles induced by various yeast strains in germfree mice. To this end, germfree C57B/6 mice were colonized with, *R. aurantiaca* and *S. cerevisie* for 6 weeks; RNA was collected from the colon of each animal and used for microarray analysis. Colonization with *R. aurantiaca* and *S. cerevisiae* revealed differential expression in 50 genes and 34 genes, respectively, when compared to germfree controls. When both fungal cohorts were analyzed in conjunction and compared to germfree animals, 356 genes were significantly different. Expression analysis between the two fungal cohorts revealed 59 genes differentially expressed (Figure 4. A). When performing a pairwise comparison of each fungally-associated cohort with germfree controls, the membrane trafficking protein Synaptotagmin 10 (Syt10), known to function as a Ca^{2+} sensor necessary for neurotransmitter and hormone release, was the most up- or down-regulated gene, demonstrating an 11.5 fold increase in *R. aurantiaca* mono-association over germfree controls. When comparing *S. cerevisiae* to germfree, a 10.24 increase in Olfactory receptor 1012 (Olfr1012) was observed and interestingly, Olfr1012 was one of very few genes that were differentially expressed between treatment groups (Figure 4. B, C). Olfactory receptors constitute the largest multigene family in mammals. Mouse and human genomes have over 1500 and 900 genes, respectively; here we report that the yeast effects the expression of 34 olfactory receptors that are significantly different from controls, with three being differentially expressed between the two treatment groups (Figure 4. E).[37] Also of note was the stress-related survival factor, Erythroid differentiation regulator-1 (ERDR1), which has shown to have antimetastic effects in melanoma.[38] Up-regulation of ERDR1 was present in both mono-associated cohorts (Figure 4. D).

Previous studies have shown inflammatory processes as the most common biological outcome when germfree mice were mono-colonized with segmented filamentous bacteria (SFB) and demonstrate substantial fold increases over control mice.[39] As the host immune system

expresses a variety of receptors that function to detect yeast, we hypothesized that colonization of animals with yeast would also induce a very robust immune response. By comparison, the largest fold increase in any gene was only 11.5 fold. Surprisingly, MLNs from mono-colonized animals revealed no differences in T-cell subsets. Although several innate programs were affected by mono-colonization, we could find no evidence of an adaptive immune response. Further corroborating this finding, KEGG pathway analysis revealed that neither inflammation nor adaptive immunity was not among the top ten biological processes affected when germfree mice were mono-colonized with our two species of yeast. Rather, multiple pathways associated with metabolism and digestion were highly influenced by the mono-colonization of yeast (Figure 5. A-C). Interestingly, pathways relating to tight junction regulation were also among the most influenced between the two treatment groups. To further explore this effect, we employed Ingenuity, a functional genomic program, to better elucidate the biological relevance of our microarray data. Ingenuity analysis confirmed the absence of an inflammatory and adaptive immune response and highlighted pathways involved in epithelial adherens and actin cytoskeleton signaling (Figure 5. D-F).

It appears that yeast was treated much more like a food source, rather than a potentially infectious agent. Multiple pathways associated with metabolism and cellular adherens, charged with regulating the flux of ions or nutrients across a semipermeable membrane, like that of the colon, and the dearth of immune effectors, provide evidence for this observation. As barrier function was a common denominator in our analysis, it is important to articulate that barrier function can be divided into two separate categories: (1) barrier defense and (2) barrier integrity.

(1) Barrier defense can be defined as the litany of AMPs employed to thwart invading pathogens as well as bridle commensal organisms that veer beyond the mucus lining of the GI. Global expression analysis revealed a marked increase in antimicrobial peptide (AMPs) programs upon mono-colonization, specifically Regenerating islet-derived 3-beta (REG3β) and REG3γ (Figure 6. A). AMPs provide an initial line of defense to bacterial, fungal, and viral pathogens and have been shown to modulate microbial communities in the gut.[18,40] These genetic programs were all significantly increased in animals mono-associated with *R. aurantiaca*,

while the same AMPs were only modestly increased in the *S. cerevisiae* group (Figure 6. B, D). Quantitative real-time polymerase chain reaction (qPCR) for REG3β and REG3γ showed an insignificant trend in the amplified transcript levels of *S. cerevisiae* and *R. aurantiaca* (Figure 6. C, E). These data demonstrate that commensal/environmental yeast when mono-colonized in a germfree mouse induces a genetic signature aimed at eliminating and/or controlling a diverse repertoire of microbes, which is crucial for the maintenance of intestinal barrier defense.

(2) Barrier integrity might best be described as the intricate network of transmembrane (claudins, occludin) and peripheral membrane (zonula occludins) proteins that work to control the movement of solutes inter- and intracellularly, based on size and charge selectivity.[41] Evaluating the gene expression of these three protein families, we observed no differential gene regulation; both yeast seem to induce the same genetic program with *R. auarntiaca* intensifying the concordant effect (Figure 7. A). In order to delineate the tortuous combination of these proteins, we focused specifically on three claudins: 2, 7, and 12 which have been reported to enhance barrier function and whose disruption has been associated with IBD; two of which, Cldn 7 and 12, were significantly reduced in both treatment groups when compared to germfree controls (Figure 7. B).[41] qRT-PCR from two independent experiments shows no change in claudins 7 and 12 transcripts; however, we show that colonization with *R. aurantiaca* produced a significant increase in Claudin 2 expression (Figure 7. C-E). Claudin 2 is a pore forming protein necessary for paracellular transport of cations; moreover, claudin 2 expression has been shown to regulate colonocyte proliferation and ameliorate DSS colitis, thus providing one explanation as to why animals treated with *R. aurantiaca* suffer less disease than their *S. cerevisiae* counterparts.[41,42] Several investigators have begun to evaluate the effect of the microbiota on barrier permeability and specifically claudin expression; however, no one has asked how yeast may effect the formation of these junctional complexes. Our study provides evidence that yeast induce a genetic signature that influences barrier integrity.

These two modalities, barrier defense and barrier integrity, work together to maintain epithelial homeostasis, yet within each modality exists several parameters able to influence its function. Genetic predispositions, for example, that augment the microbial composition in the

gut, are associated with Intestinal permeability and increase susceptibility to disease.[14,26,41] How individual members of the microbiota influence barrier function, however, is poorly defined.[41] Conversely, it is well established that the microbiota has a significant impact on host metabolism in the gut; moreover, metabolic by-products have been clearly associated with intestinal permeability.[43] How commensal yeast effect these biological processes remains largely unexplored; therefore, we sought to determine if the differences in colonic histology observed during chemically induced colitis were a result of host metabolism.

Mono-colonization with S. cerevisiae Leads to Excess Purine Degradation Products, Including Uric Acid

Fungi are known inhabitants of the mammalian GI; however, their role in digestion and influence upon metabolic pathways has not been established. To investigate the impact fungi have on metabolic processes within the colon, we analyzed the feces for 126 metabolites by liquid chromatography mass spectrometry (LC-MS) from germfree, mono-colonized (yeast), and specific pathogen-free (SPF) animals. Hierarchical clustering analysis reveals a significant difference in the fecal metabolic profile (FMP) of germfree and SPF animals (Figure 8. A). To further explore the metabolomics of mono-colonized animals, we asked if the mono-colonization with yeast results in a FMP that closely resembles that of germfree or SPF mice or an intermediate between the two. Moreover, we were interested in the presence of metabolites that may result in adverse pathology to the host.

Principal component analysis (PCA) reveals that indeed mono-colonization with two different phyla of yeast produces a FMP that closely resembles that of germfree mice (Figure 8. B). However, there was significant variation between mono-colonization and germfree cohorts; we observed significant differences in 7.1% (9 out of 126) of fecal metabolites analyzed (Figure 8. C). Five of the 9: Mannitol, ribitol/xylitol, egosterol, shikamate, and quinic acid, were the most significantly different metabolites and the primary drivers of the differences seen between germfree and mono-colonized animals (Figure 9. A). Furthermore, these metabolites were also

reduced in SPF animals, indicating that commensal fungi may be important for mediating these metabolic pathways. In addition to differences seen between mono-association and germfree animals, we also observed significant differences between the two treatment groups. Animals mono-colonized with *S. cerevisiae* show a marked increase in hypoxanthine and xanthine over both germfree controls and the *R. aurantiaca* cohort (Figure 9. B). SPF animals, however, had nearly 200 fold more of these metabolites (Figure 9. B). We then noticed that uric acid in SPF animals was considerably reduced while germ free and mono-colonized animals remained elevated (Figure 9. B). Animals colonized with *S. cerevisiae* remained significantly increased over germfree controls (Figure 9. B). Interestingly, these three metabolites comprise the major constituents of the purine degradation pathway (Figure 9. C).[44] Although mice metabolize uric acid further, higher primates do not and uric acid has been associated with multiple inflammatory disorders.[45]

These data indicate that, as suspected, bacteria bear the majority of the metabolic capacity of the microbiota. Nonetheless, we demonstrate that two different phyla of yeast have drastic effects of the FMP with *S. cerevisiae,* resulting in significantly elevated levels of uric acid. How differences in microbial composition influence the production of specific metabolites and how those metabolites then affect downstream physiology is an emerging topic of microbiota research.[46] Uric acid is a potent antioxidant and is correlated with both deleterious and ameliorating effects. Our study implies that certain species of yeast may have the ability to potentiate uric acid, leading to disrupted barrier function.

Uric Acid Aggravates Disease and Increases Intestinal Permeability During DSS Colitis

The disease phenotype we established, in conjunction with our microarray analysis, implied that yeast affects pathways associated with metabolism that can have deleterious effects on epithelial integrity. The metabolomics profile we performed identified three independent metabolites existent in the same metabolic pathway; therefore, we asked if uric acid alone could recapitulate the effects seen during DSS colitis. To address this, we orally gavaged uric acid at a

concentration of 10mg/ml during DSS colitis and evaluated the colons for histology as we had previously. In addition, we performed a functional assay, using FITC-dextran a model antigen, to assess the effect of uric acid on intestinal permeability. And lastly we used a chemiiluminescent assay to detect uric acid in the feces and serum from these animals.

Here we report that treatment with uric acid during DSS colitis results in aggravated colonic histology demonstrated by complete destruction of epithelial structure and crypt loss (Figure 10. A). Blind scoring of H&E stained colons confirms that uric acid leads to exacerbated pathology (Figure 10. B). Remarkably, treatment with uric acid led to increased intestinal permeability as measured by FITC flourophores in the serum (Figure 10. C). As mice contain the enzyme uricase, which reduces uric acid, we assayed both the serum and feces of these animals to ensure that our delivery system provided substantial uric acid beyond what mice are biologically capable of breaking down. We show that uric acid levels were significantly elevated in the feces; however, we were not able to show an increase in the serum (Figure 10. D).

Our previous findings show that particular members of the mycobiome are capable of augmenting uric acid levels in the gut; thus, the discovery that uric acid produces adverse pathology during experimental colitis and results in increased intestinal permeability is extremely exciting. To our knowledge, this is the first time a specific metabolite has been shown to disrupt the colonic mucosa and has profound implications for inflammatory disorders of the bowel. Several medications known to inhibit purine degradation are currently used to resolve inflammatory symptoms in CD.[47] These data provide evidence that the microbial composition of the gut can indeed worsen an underlying inflammatory condition and in the future may provide additional options for therapy in these patients.

Saccharomyces cerevisiae Increases Intestinal Permeability and Serum Uric Acid During DSS Colitis

In order to sufficiently connect the phenotype displayed by *S. cerevisiae* during DSS colitis and the result seen when animals were supplemented with uric acid during DSS colitis, we asked if treatment with *S. cerevisiae* during DSS colitis results in increased serum and fecal uric

acid levels, and if *S.cerevisiae* treatment is associated with increased intestinal permeability. Therefore, we repeated our DSS experiments using our two model yeasts as before and assayed for uric acid in the serum and feces. We also repeated our functional assay for intestinal permeability to evaluate if *S. cerevisiae* could produce the leaky gut phenotype seen when animals were given uric acid.

Here we show that animals orally gavaged with *S. cerevisiae* during DSS colitis have significantly more uric acid in their serum than do DSS controls and animals treated with *R. aurantiaca* (Figure 11. A). No differences were seen in uric acid levels in feces (Figure 11. B). Strikingly, animals treated with *S. cerevisiae* during disease exhibited a significant increase in intestinal permeability when compared to DSS controls and animals treated with *R. aurantiaca* (Figure 11. C). Due to the leaky gut phenotype, we wanted to investigate the junctional proteins that may be responsible for the observed leaky gut phenotype. qRT-PCR analysis depicted a significant increase in Cldn7 when animals were treated with *R. aurantiaca* (Figure 11. D). Cldn7 is known to be expressed in the small and large intestine with high variability and, like Cldn2, acts as a cation pore for paracellular transport.[48] Furthermore, it has been shown to be important for E-cadherin regulation and overexpression of Cldn7 was shown to enhance adhesiveness of esophageal cells in vitro.[48] Our data therefore suggests that members of the mycobiome can modulate claudin expression in intestinal epithelial cells through the action of host metabolites.

Here we provide a mechanistic rationale for the phenotype observed when animals are supplemented *S. cereivisae* during an experimental model of colitis. As many patients with IBD are treated with antibiotics as a form of therapy, this can, and has been documented to lead to an overgrowth of the mycobiome.[15] Furthermore, xanthine oxidase inhibitors such as allopurinol, which prevent the formation of uric acid, have shown to improve clinical outcomes in CD patients.[47] Hence, our study in combination with previous literature, suggests that an increased abundance of the mycobiome can lead to metabolic differences within the microenvironment of the gut that can negatively affect the intestinal mucosa and prove detrimental to the host.

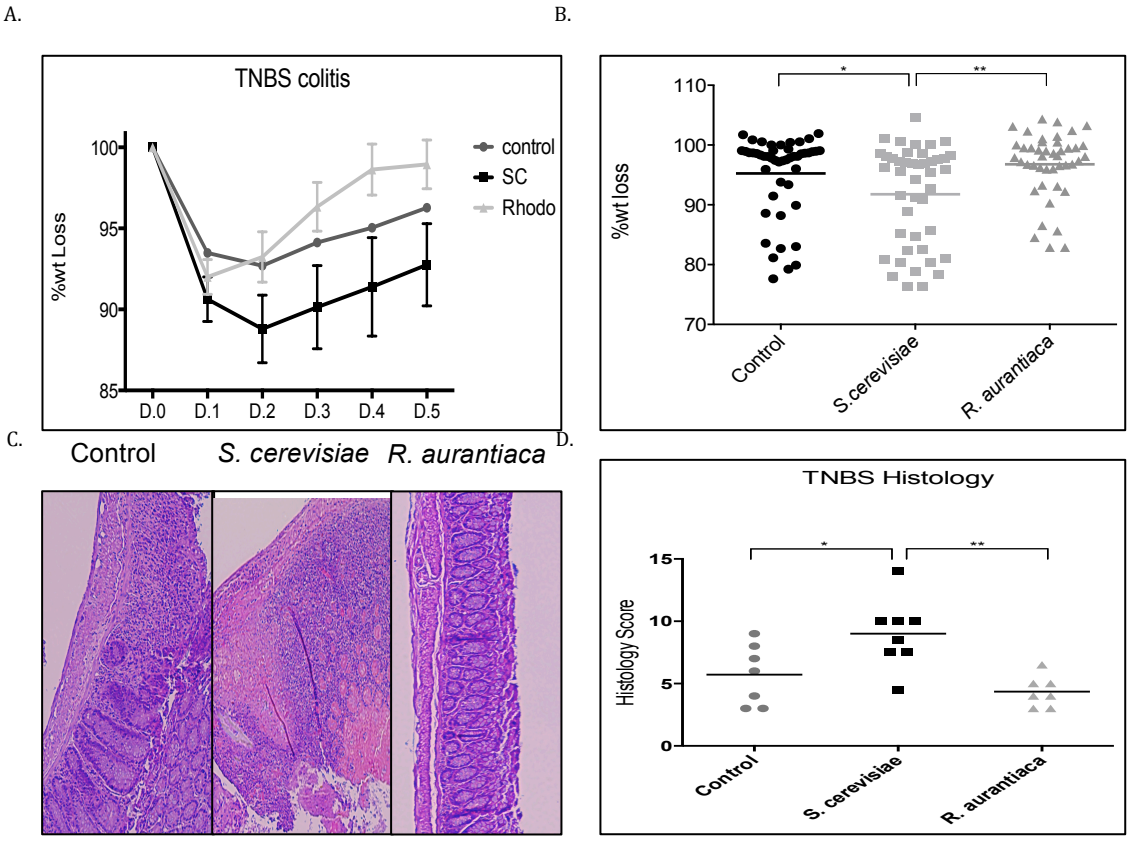

Figure 1. *Saccharomyces cerevisiae* exacerbates pathology during TNBS colitis. A) Average percent weigh loss from three independent experiments. Error bars removed from control group for clarity. B) Percent weight loss for each animal from days 2 to 5 were compared by a students t-test. Animals treated with *S. cerevisiae* show reduced ability to recover compared to controls and *R. aurantiaca*-treated animals. C-D) H&E staining of TNBS colons shows that animals treated with *S.cerevisiae* suffered a greater degree of crypt destruction and loss epithelial structure than controls and those treated with *R. aurantiaca.*

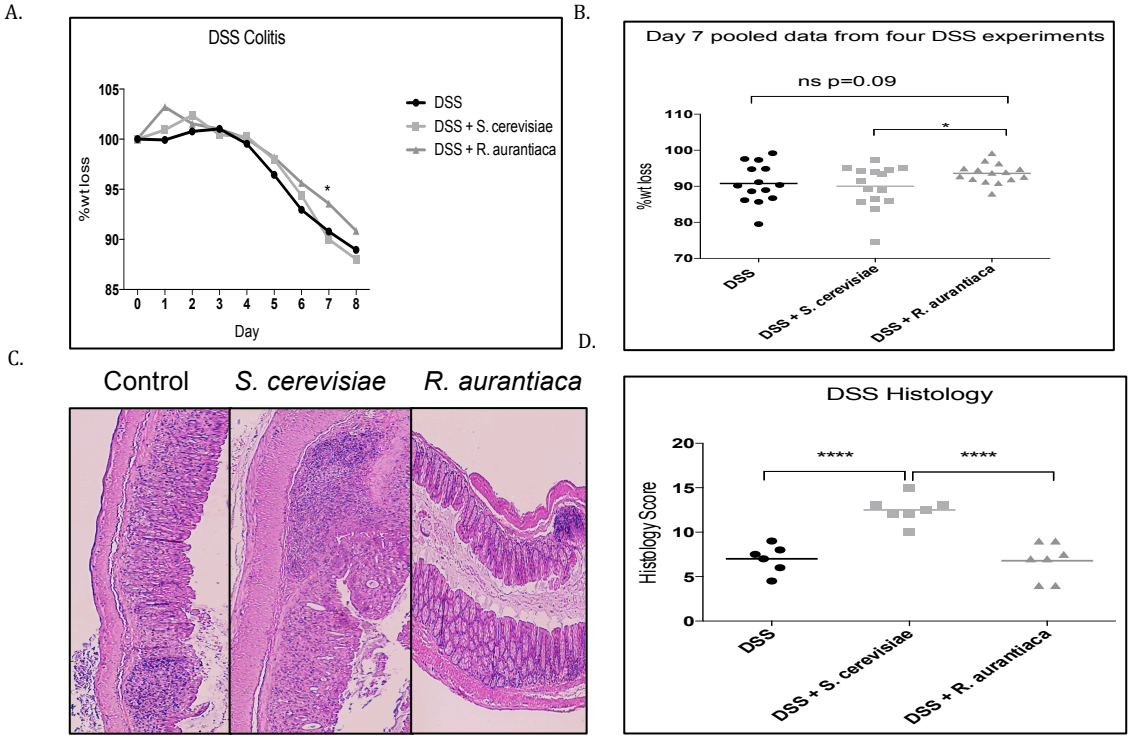

Figure 2. *Saccharomyces cerevisiae* recapitulates TNSB phenotype during DSS-induced colitis A) Average percent weight loss from four independent experiments. Asterix denotes a significant difference in weight loss in animals treated with *S. cerevisiae* compared to *R. aurantiaca*. B) Percent weight loss at day 7 from each animal over four independent experiments. Animals treated with *R. aurantiaca* lost significant less weight than those treated with *S. cerevisiae* and trended to loose less than DSS controls. C-D) H&E staining of TNBS colons shows that animals treated with *S.cerevisiae* suffered a greater degree of crypt destruction and loss of epithelial structure than controls and those treated with *R. aurantiaca.*

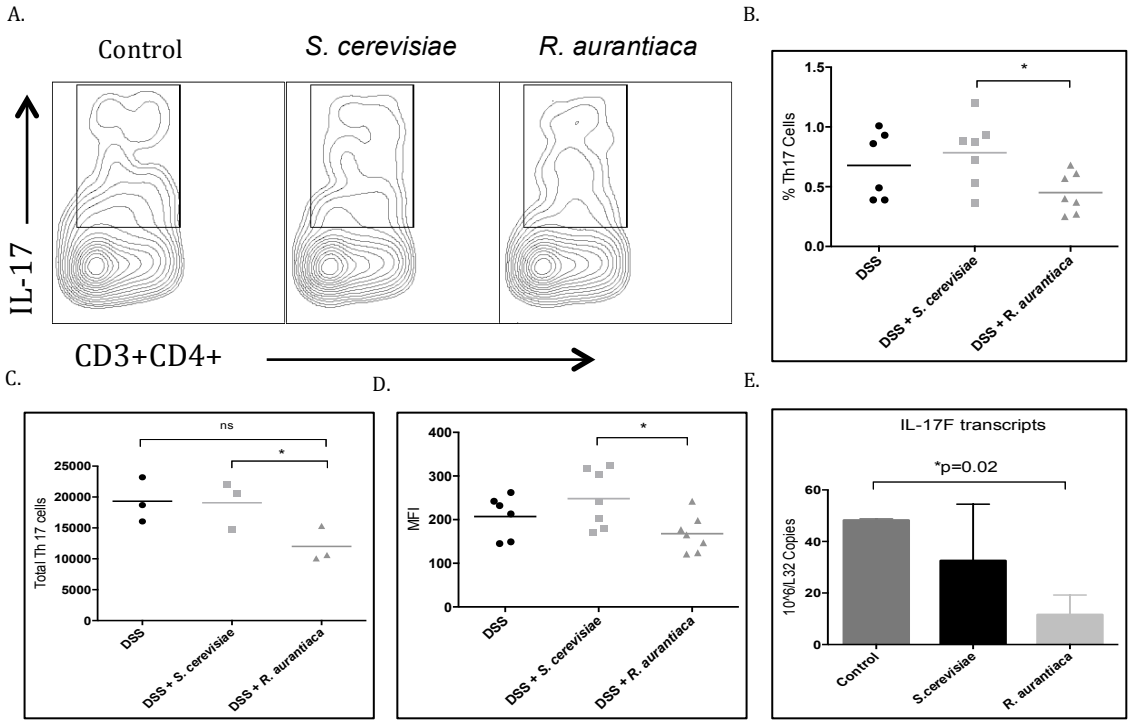

Figure 3. Treatment with yeast during DSS colitis does not induce robust inflammatory response. A) Representative flow plot and quantitative analysis showing reduced CD3+ CD4+ IL-17+ positive cells in the mesenteric lymph nodes as well as total Th17 cells (B) and Mean fluorescent intensity (MFI) (C) of animals treated with *R. aurantiaca* during DSS colitis. D) qPCR of colonic tissue from DSS animals shows significant reduction in IL-17F transcripts when animals were supplemented with *R. aurantiaca.*

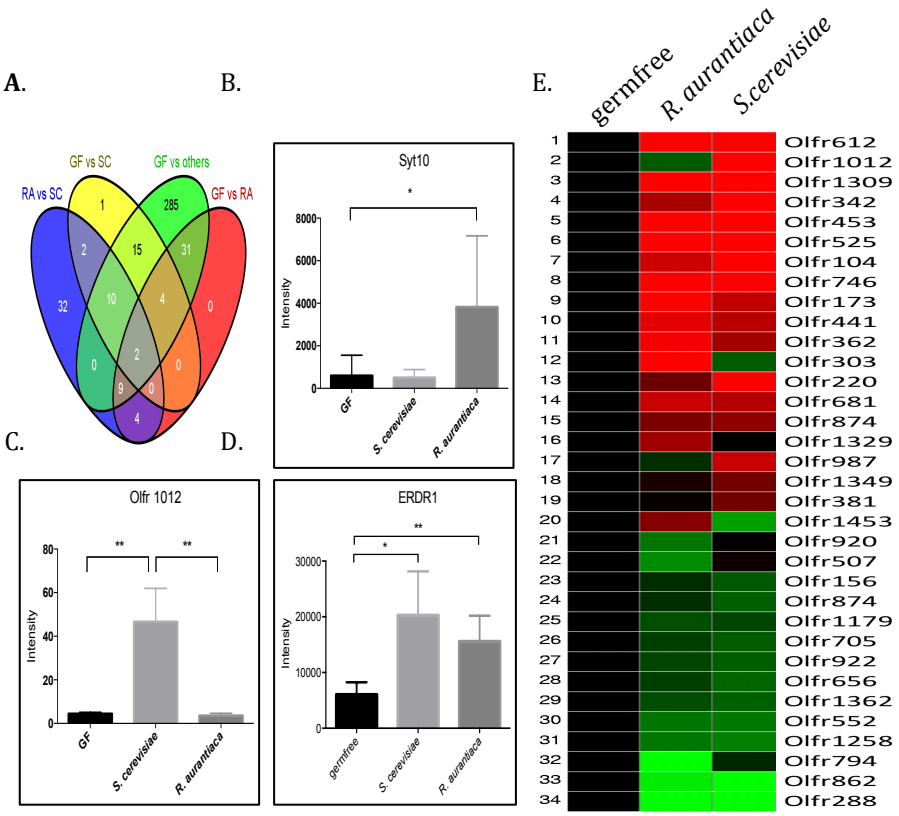

Figure 4. Germfree mice mono-colonized with commensal yeast produces transcriptome with few differences. A) Venn diagram shows differential gene expression among cohorts. B-D) Raw intensity values from the microarray analysis were linearly transformed and compared by a students t-test. (B) The Calcium sensor Synaptotagmin 10 generated the differential expression between *R. aurantiaca* and germfree controls. (C) Olfactory receptor 1012 demonstrated the greatest differential expression between *S. cerevisiae* and germfree controls; Olfr1012 was one of few genes differentially influenced by the two yeast. (D) Erythroid differentiation regulator-1, shown to be important in cell survival is markedly increased during mono-colonization with yeast. E) Heat map shows most statistically significant genes within the Olfr gene family.

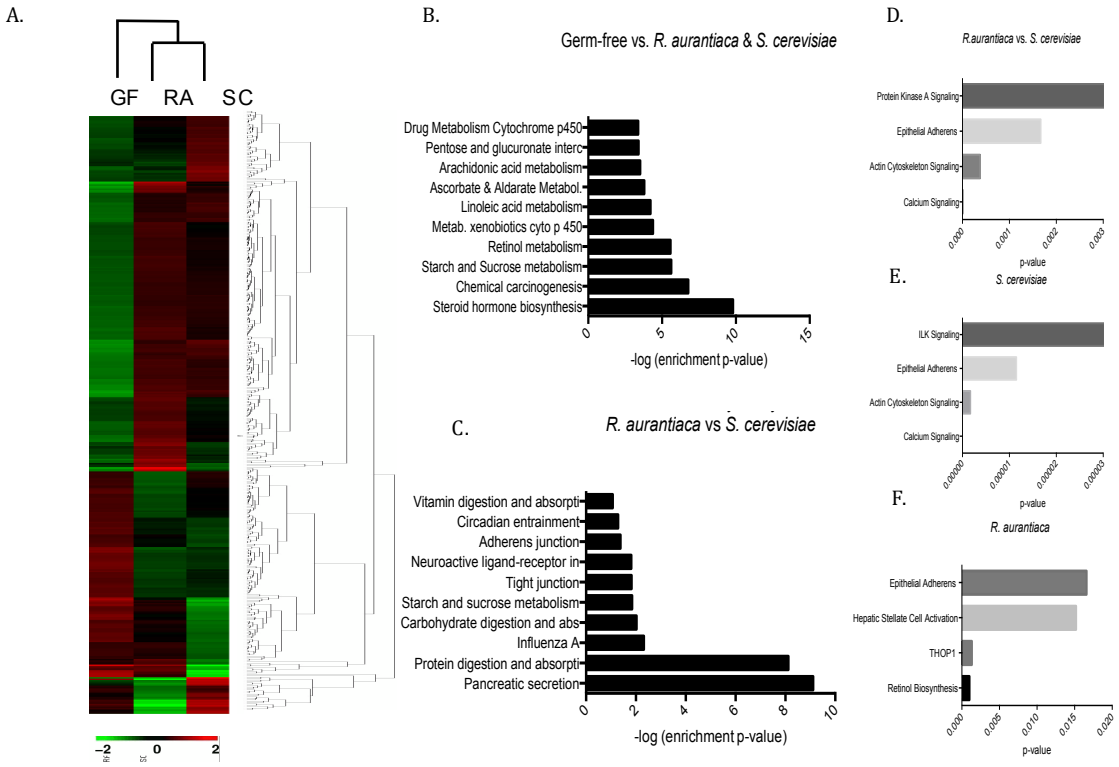

Figure 5. Mono-colonization with commensal yeast influences pathways associated with metabolism and barrier function. A) Heat map shows hierarchal clustering of differentially regulated genes between mono-associated and germfree animals. B-C) KEGG pathway analysis shows the top ten physiological pathways affected by when germfree animals are compared to both treatment groups (B) and between the two treatment groups (C). D-F) Ingenuity analysis between treatment groups (D), when animals mono-colonized with *S. cerevisiae* are compared to germfree controls (E) and when animals mono-colonized with *R. aurantiaca* are compared to germfree controls (F).

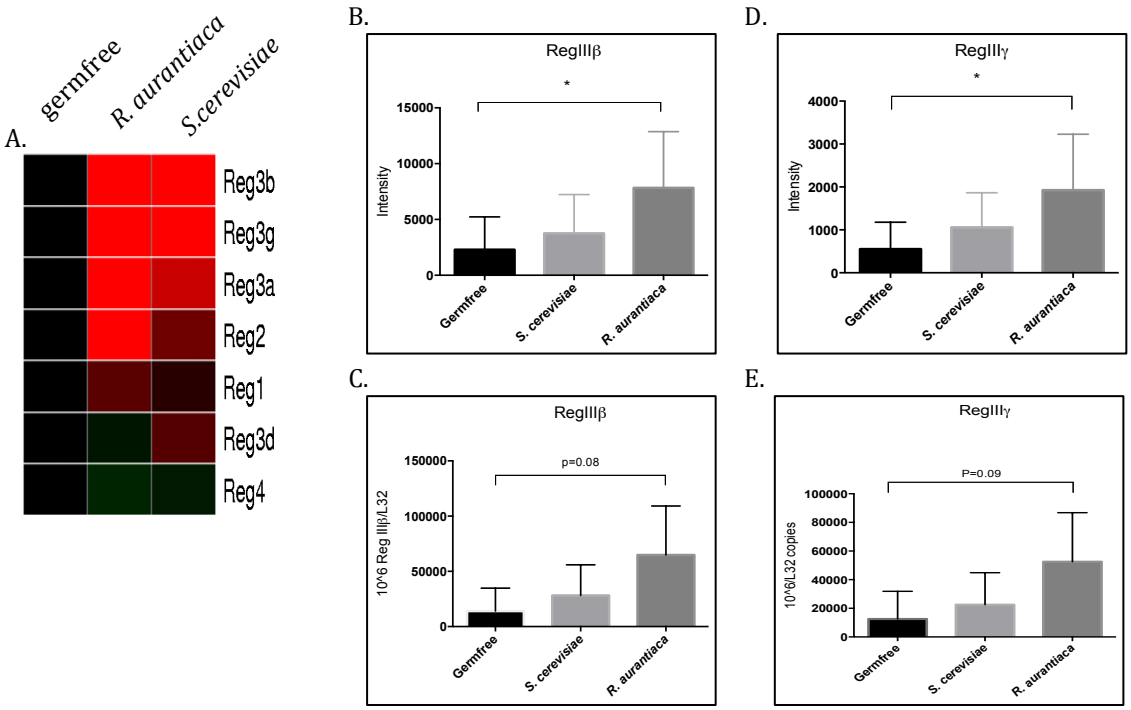

Figure 6. Commensal yeast species up-regulate an antimicrobial peptide program upon mono-colonization. A) Heat map shows global expression patterns for the regenerating islet derived gene family. B) Raw intensity values of RegIIIβ microarray analysis were linearly transformed and compared by a student t-test. C) qRT-PCR performed on colonic tissue from mono-colonized animals verifies increase in RegIIIβ. D-E) same as (B) and (C) for the RegIIIγ.

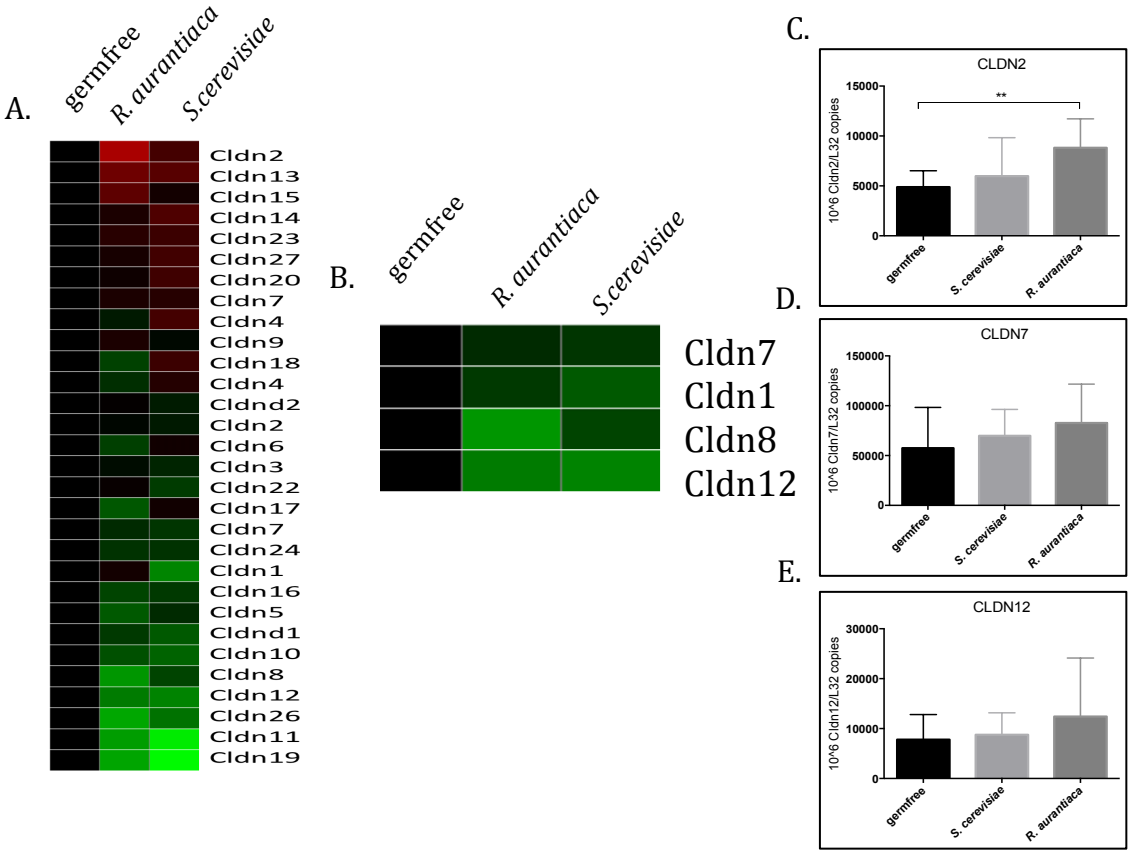

Figure 7. Commensal yeast influence barrier integrity modulating transcripts of junctional proteins. A) Heat map shows expression of claudin proteins during mono-colonization. B) Heat map shows statistically significant (ANOVA) differences in claudin proteins during mono-colonization. C-E) qPCR of Cldn expression from colonic tissue. Expression values were compared by students t-test. Germfree/*R. aurantiaca* cohorts: n=8; *S. cerevisiae*: n=7.

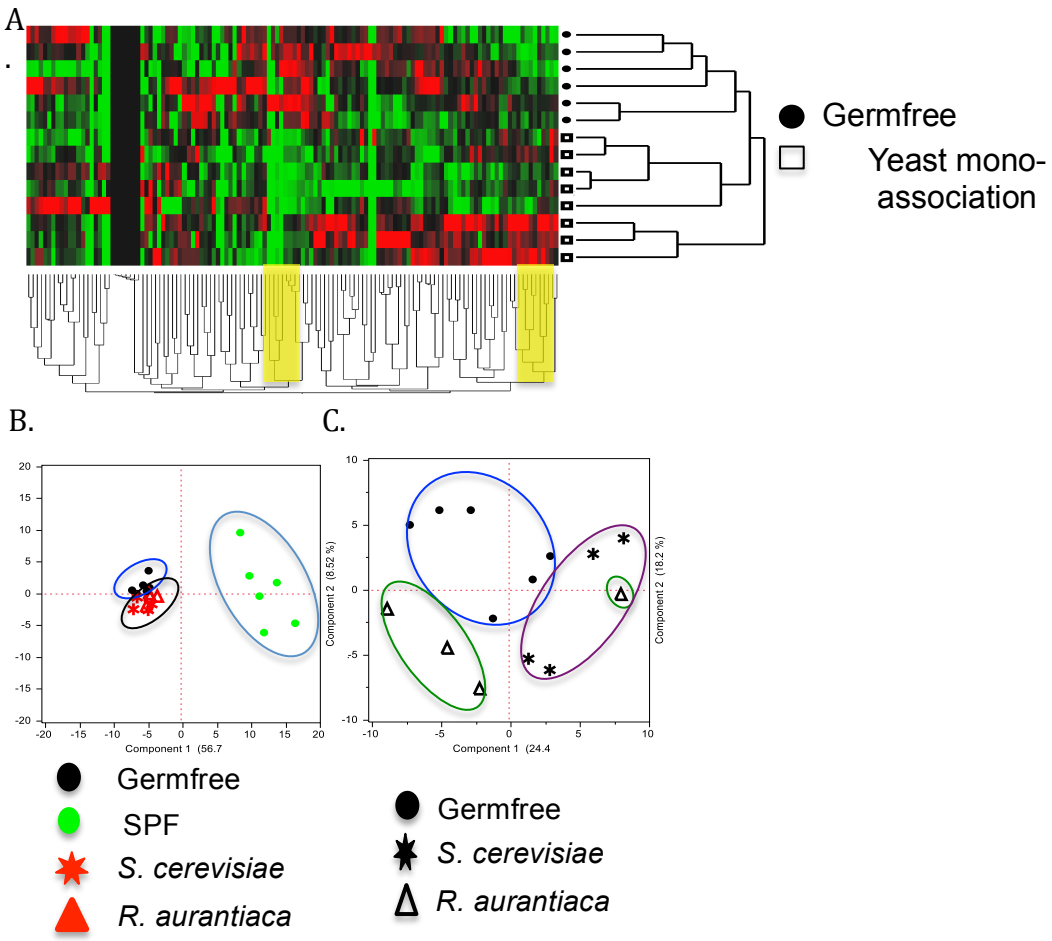

Figure 8. Conventional colonization and mono-association with two different yeast species biases fecal metabolic profiles. A) Two way hierarchal clustering analysis based on colonization status and fecal metabolite abundance. B) Principle component analysis (PCA) of fecal metabolite abundance between germfree, SPF, mono-colonization with *S. cerevisiae* and *R. aurantiaca.* C) PCA of fecal metabolite abundance between germfree and each mono-colonized cohort.

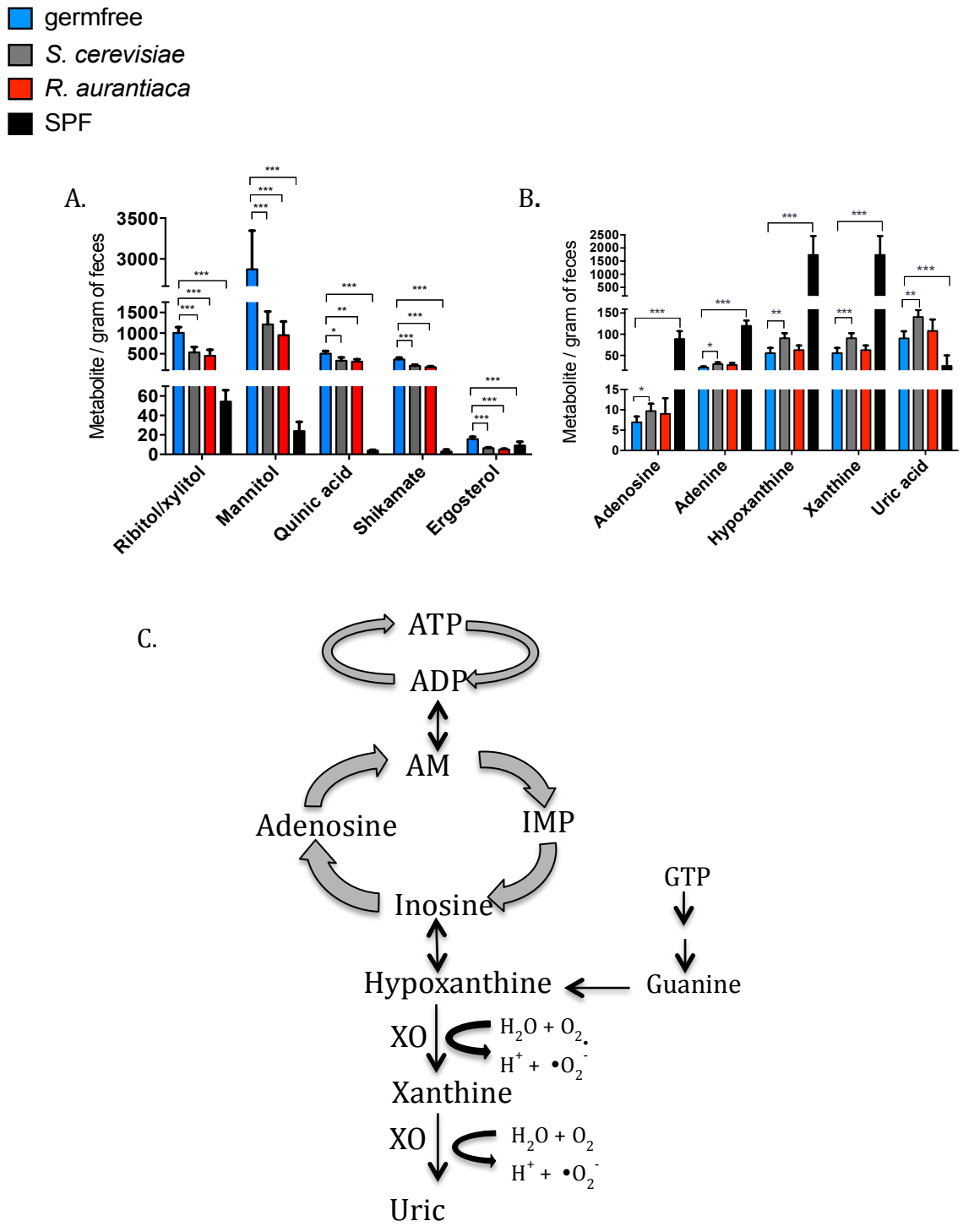

Figure 9. *S. cerevisiae* influences metabolites in the purine degradation pathway. A) Shows fecal abundance of metabolites reduced in SPF and mono-colonized animals. B) Shows fecal abundance of metabolites in the purine degradation pathway. C) Purine degradation pathway, adapted from Marro et al. Xanthine oxidase (XO)

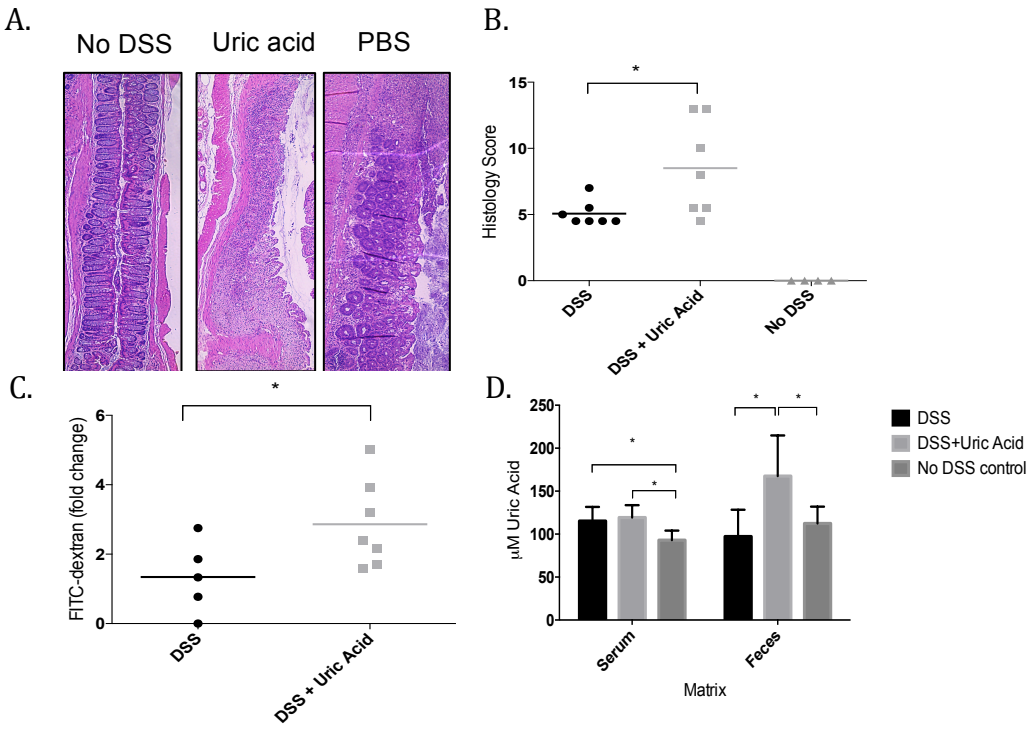

Figure 10. Addition of uric acid during DSS colitis results in colon pathology and increased barrier permeability. A) H&E stained colon sections. B) Histology scores from H&E stained colons show worse disease when animals received uric acid during DSS colitis. C) Shows fold change of FITC-dextran in the serum. Values were normalized to negative controls that received FITC-dextran but not DSS. D) Measurement of uric acid in the feces and serum of DSS, DSS+Uric acid, and no DSS control animals.

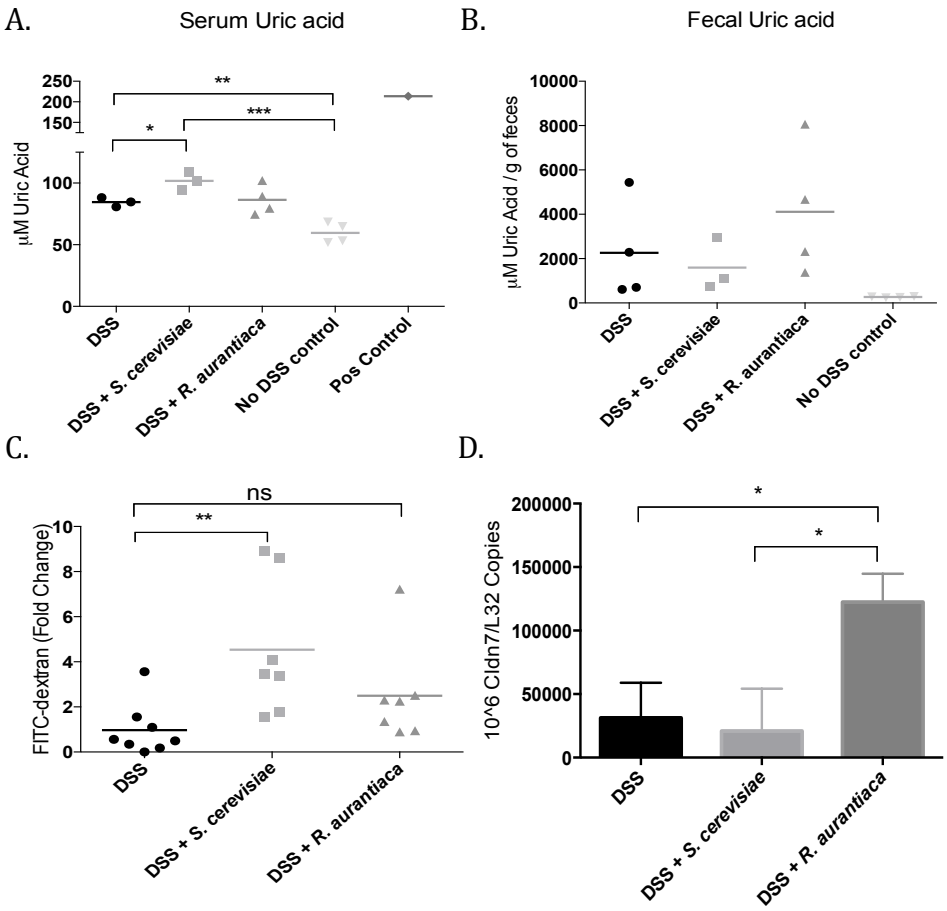

Figure 11. *S. cerevisiae* increases serum uric acid levels and intestinal permeability. A) Shows serum uric acid levels. B) Shows fecal uric acid levels. C) Shows fold change of FITC-dextran in the serum. Values were normalized to negative controls that received FITC-dextran but not DSS. D) qRT-PCR for claudin 7 expression performed on colon tissue

DISCUSSION

Summary

Despite the fact that environmental fungi live almost exclusively as obligate mutualists, we know very little about their functions in the gut or how individual species contribute to host health. Here we show that two distinct phyla of yeast differentially influence susceptibility to disease by augmenting the metabolic profile in the gut. Specifically, we demonstrate that treatment with *S. cerevisiae,* but not *R. aurantiaca,* leads to increase abundance of metabolites in the purine degradation pathway, including serum levels of uric acid. Moreover, animals treated with *S. cerevisiae* were more susceptible to disease as measured by colonic histology and exhibited a higher degree of intestinal permeability, which has been associated with several inflammatory disorders of the bowel.[49] Additionally, we were able to recapitulate these effects when we supplemented uric acid to animals during DSS colitis. Thus, our data imply that some yeast have the ability to influence the metabolic capacity of the host, which we show can result in metabolites that can further exacerbate disease.

Uric Acid, an Evolutionary Conundrum

Uric acid is most commonly known and has long been recognized as the etiological agent of Gout.[50,51] However, uric acid has a long history and has been implicated to both ameliorate disease and promote inflammation.[50,52] Uric acid is a diprotic acid and a strong reducing agent capable of binding Fe^{3+} and inhibiting lipid peroxidation.[52-54] Most animals contain the enzyme uricase, which breaks down uric acid to the more soluble compound, allantoin; however, during primate evolution, nonsense mutations have made this gene nonfunctional.[53] The lack of uricase in higher primates makes uric acid the final breakdown product in purine metabolism.[55] Interestingly, the loss of uricase coincides with the inability to synthesize ascorbic acid, another powerful reducing agent, which some have proposed as a compensatory

mechanism by increasing plasma uric acid levels and thereby reducing potentially damaging reactive oxygen species.[53]

Gout is an inflammatory disorder of purine catabolism resulting in uric acid crystals, known as monosodium urate (MSU), depositing in the joints and periarticular tissues.[51] Since this discovery over two hundred years ago, the biological significance of uric acid has been studied immensely. When cells undergo damage, they release signals known as damage-associated molecular patterns (DAMPS), alerting the surveying immune system of their demise.[56] Uric acid has shown to be a DAMP and a powerful adjuvant released during cell injury and contributes to the immunostimmulatory effect of surveying immune cells.[55,57] Furthermore, it has been demonstrated that MSU is capable of up-regulating co-stimulatory molecules on dendritic cells and stimulating cytotoxic T lymphocytes (CTLs) in vitro.[55] Additional studies have reveled that MSU binds to the NALP3 inflammasome resulting in IL-1β and IL-18 production.[50] Macrophages derived from mice, deficient in the molecular constituents of the inflammasome, were unable to produce these cytokines when incubated with MSU and exhibited a diminished neutrophil influx during a MSU-induced model of peritonitis.[50] Hence, uric acid and its derivatives have numerous immunomodulatory applications. Forty-five years ago, Edozein et al. demonstrated that feeding young males *Candida utilis* increased both serum and urine uric acid levels to levels associated with gout.[58] More recently, Hoffman et al. have shown that a diet rich in carbohydrates can lead to an increased fugal burden in the GI.[3] These data in combination with the observation that CD patients have greater abundance of *Candida* spp. argue heavily that factors resulting in a greater abundance of the mycobiome can affect the metabolic outcome of the host, which in genetically predisposed individuals can worsen disease.

The development of radical oxygen and nitrogen species during inflammation has proven an effective mechanism for eliminating pathogens. However, during aberrant immune responses like that in IBD, these inflammatory mediators can result in tissue damage.[59] During homeostatic conditions, cells are able to combat these radical species with a host of antioxidant defenses, including urate (the ionic form of uric acid).[59] Under constant oxidative stress, like that seen in chronic inflammation, tissues become depleted of these antioxidant defenses,

exacerbating the pathology.[59] In a cohort of CD and UC patients, urate, along with several other antioxidants, was shown to be significantly reduced in the colonic mucosa of these patients.[59] Another disease of chronic inflammation, Multiple Sclerosis (MS), results from inappropriate adaptive immune responses to the central nervous system. Interestingly, serum uric acid levels were markedly reduced in a cohort of MS patients and a clear inverse correlation between gout and MS was determined by data mining the records of over 20 million patients.[52] The nitrogen radical, peroxynitite, has been associated with lesions in the brains of MS patients.[60] Uric acid, however, has been shown to bind and inactivate peroxynitrite.[61] Using a chronic model of experimental allergic encephalomyelitis (EAE), Hooper et al. demonstrated that therapeutic doses of uric acid were capable of inhibiting the onset of clinical disease as well as rescuing animals suffering from clinical symptoms.[52] Thus, a delicate balance exists between uric acids ability to scavenge potentially harmful radical oxygen species and its ability to cause further tissue pathology.

Collectively, these studies provide further evidence to the biological significance of uric acid and its potential to be used in medicine. The discovery that MSU is a strong adjuvant and potent stimulator of CTLs could one day lead to better vaccine development, as current adjuvants are not capable of activating $CD8^+$ T lymphocytes.[55] Furthermore, the mutual exclusion seen between gout and MS testifies to uric acids' strong reducing ability and why the loss of uricase has contributed greatly to the speciation and health of *Homo sapiens.*[53] Here we report that *Saccharomyces cerevisiae* contributes to increased uric acid levels in the feces of mono-associated animals and serum of SPF animals supplemented with *S. cerevisiae*, presumably due to colonocyte injury and subsequent release of urate as an antioxidant defense or uptake of fungal metabolites, which are then shunted into the purine salvage pathway (Figure 12). Further investigation will provide additional insights on yeast and their contribution to metabolism. Selecting for a mycobiome that does not result in increased uric acid levels may one day prove to reduce the chronicity seen in inflammatory disorders such as gout and IBD.

Limitations of Our Study

One limitation of our study was the lack of a bacterial control in our mono-colonization experiment. Though we were able to glean valuable insights into the contribution of fungi both immunologically and metabolically from this study, the lack of a mono-colonized animal with a commensal bacterium restrained our ability to make comparisons between eukaryotic and prokaryotic contributions. Furthermore, due to time and technical constraints we only evaluated mesenteric lymph nodes for innate and adaptive immune responses. It is possible that the colonic lamina propria will reveal further immune pathology in animals treated with *Saccharomyces cerevisiae*. Furthermore, in our flow cytometry, we did not perform any staining for neutrophils; however, these cells have been shown to be important for resistance to mucosal invasion from fungi.[62]

Although we were able to detect a significant difference in uric acid levels from our metabolomics data, these data are difficult to translate to clinical or relevant concentrations of uric acid as these values were normalized by weight to conventionally colonized animals. Moreover, the metabolomics data were performed on feces and not serum; to date, fecal levels of uric acid have not been described to predict or be associated with any disease phenotypes. Regardless, the observation that one phyla of yeast contributes to greater cellular insult, resulting in worsened colonic histology, increased intestinal permeability and elevated levels of serum uric acid implies that individual fungi, despite their low numbers in the gut, impact host health from a metabolic perspective.

Future Directions

These experiments have shed new light on the mycobiome and have opened the door for future investigators to pursue multiple avenues regarding fungi and how they contribute to host health and the microbial ecology of the gut. In order to completely evaluate how fungi contribute to host immune development within the gut, an exhaustive appraisal of immune cells including T cells, B cells, dendritic cells, macrophages, and neutrophils from multiple locations such as the colonic lamina propria, small intestine lamina propria, mesenteric lymph nodes, and

peyers patches should be performed in germfree mice mono-colonized with known commensal fungi individually and in combination with restricted flora.

To assess how the commensal fungal population contributes to disease severity, experiments utilizing chemically induced colitis should be executed in animals treated with antifungal drugs and evaluated for immune effectors and histology. Establishing the differences in disease phenotypes in the absence of a fungal population compared to controls will further elucidate how fungi add to chronic disorders of the bowel. Moreover, clear taxonomic differences have been demonstrated in patients with CD and UC; however, no one has looked at the taxonomic dynamics of fungal ecology during IBD. At a minimum, explication of this will provide an additional layer of knowledge to the overall microbial ecology of the microbiota and its benefits and detriments to the host.

Colonization resistance to intestinal pathogens is one of the greatest benefits afforded mammalian hosts by the presence of an intact microbiota.[22] Several studies have attested to this fact, yet, how commensal fungi aid in colonization resistance has not been studied. Are we more susceptible to pathogenic organisms in the absence of fungal flora; can particular members of the mycobiome protect from intestinal pathogens? These simple yet novel studies will provide valuable information about the mycobiome and perhaps even its telos in the human gut.

Finally, given fungi's role in nutrient cycling within other complex microbial ecosystems like that of soil, their ability to decompose complex plant material that is then utilized by symbiotic bacteria within the rhizosphere suggests that their function within the GI may be to degrade food particles for use by commensal bacteria.[63] Therefore, it is logical to investigate how fungi contribute to the metabolic output of the host. Several studies recently have highlighted the importance of the microbiota in metabolism and linked individual species with this phenomenon; however, not one of them has mentioned, evaluated, or even speculated on the fungal flora and their contribution to digestive metabolism.[46,64-66] Assaying the metabolic output and digestive capability of mice with and without a fungal population will be a first step in understanding how fungi aid the host in digestion including the acquisition and consumption of nutrients. Our study, evaluating fecal metabolites in animals mono-associated with two different

phyla of yeasts, hints at how yeast may influence the abundance of certain metabolites. Given our results of elevated uric acid levels, and the phenomenon seen in MS patients as well as other diseases, it would be worth investigating if particular elements of the microbiota, perhaps yeast, are capable of modulating physiologic levels of uric acid. In addition, what is the clinical significance of uric acid in IBD and what effect does it have on intestinal permeability? And what is the consequence of uric acid levels in the feces, what are normal levels and how does it get there? Determining how components of the microbiota contribute to the generation of metabolites, like uric acid, which have both deleterious and beneficial effects will be crucial to further our understanding of the metabolic capacity of the mycobiome and its impact on human health.

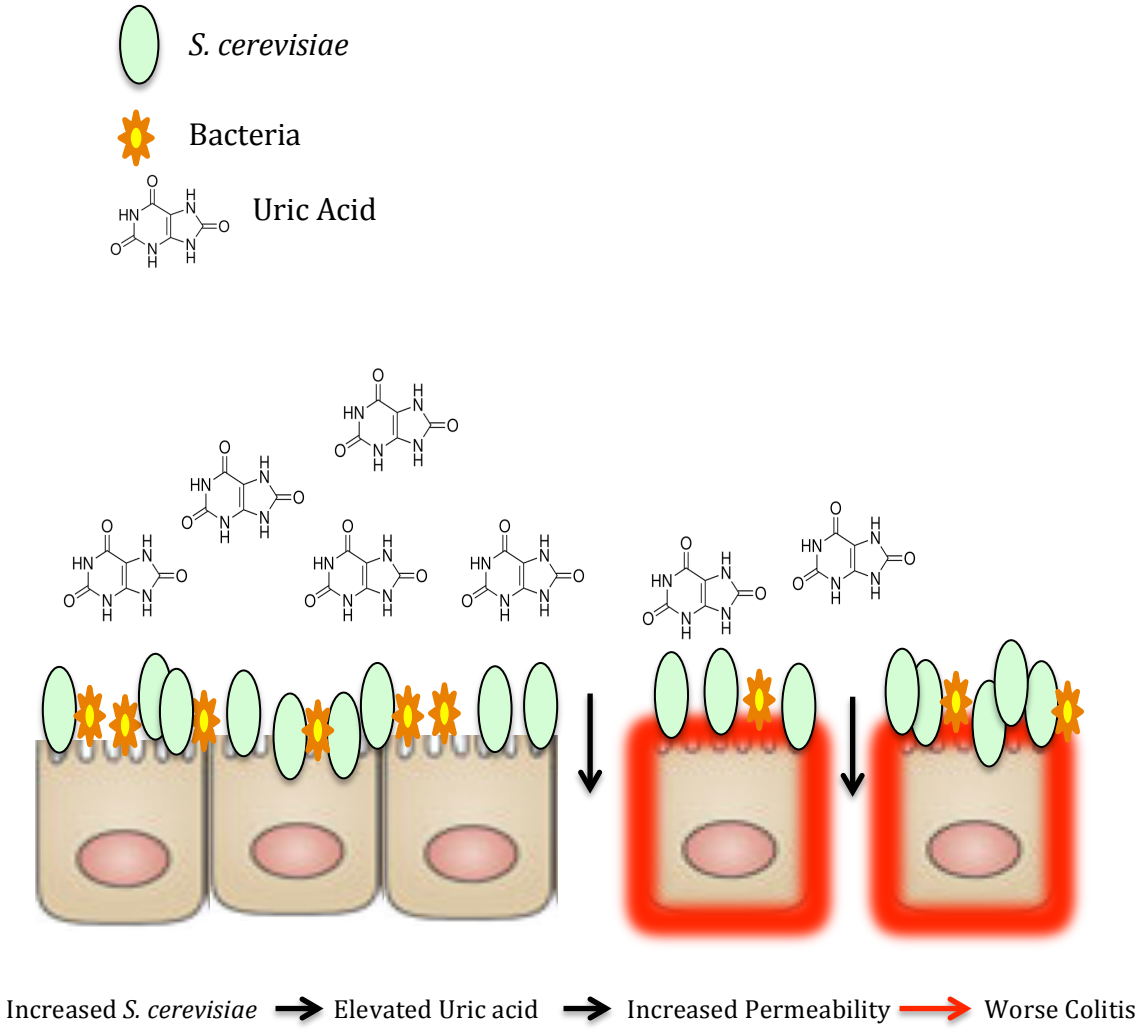

Figure 12. Overgrowth of fungal species during IBD leads to increased uric acid production and promotes colonic pathology. During homeostatic conditions, an intact microbiota, a mucus membrane, and a network of junctional proteins that control the movement of solutes across the epithelial barrier preserve epithelial integrity. During chronic inflammation, tight junction proteins are disrupted leading to increased permeability, allowing commensal organisms and food products to breach the epithelial interface resulting in increased immune trafficking, inflammatory cytokines, and humoral responses to an array of bacterial and fungal antigens. Antibiotic treatment during IBD can potentiate an overgrowth of fungi and our research suggests, that the putative increase in species such as *S. cereiviae* results in synthesis of uric acid, which further perturbs membrane function and the inflammatory process.

EPILOGUE

Microbiota research is as complex as the biological system it intends to study and represents a truly interdisciplinary field of science spanning ecology, microbiology, immunology, and biochemistry. Understanding, not only the contribution of a given kingdom, but the emergent property of the microbiome as a unique symbiotic ecosystem that is mutualisitically inseparable from its host, including the ability to manipulate it reproducibly, will be one of the greatest endeavors of scientific discovery in the 21st century.

REFERENCES

1 Minot, S. *et al.* Rapid evolution of the human gut virome. *Proceedings of the National Academy of Sciences of the United States of America* **110**, 12450-12455, doi:10.1073/pnas.1300833110 (2013).

2 Iliev, I. D. *et al.* Interactions between commensal fungi and the C-type lectin receptor Dectin-1 influence colitis. *Science* **336**, 1314-1317, doi:10.1126/science.1221789 (2012).

3 Hoffmann, C. *et al.* Archaea and fungi of the human gut microbiome: correlations with diet and bacterial residents. *PloS one* **8**, e66019, doi:10.1371/journal.pone.0066019 (2013).

4 Ahern, P. P., Faith, J. J. & Gordon, J. I. Mining the human gut microbiota for effector strains that shape the immune system. *Immunity* **40**, 815-823, doi:10.1016/j.immuni.2014.05.012 (2014).

5 Mazmanian, J. L. R. a. S. K. Inducible Foxp3+ regulatory T-cell development by a commensal bacterium of the intestinal microbiota. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 12204-12209 (2010).

6 Rosner, J. L. Ten Times More Microbial Cells than Body Cells in Humans? *Microbe* **9** (2014).

7 Ann Reid, S. G. Human Microbiome FAQ. *American Academy of Microbiology* (2013).

8 Backhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A. & Gordon, J. I. Host-bacterial mutualism in the human intestine. *Science* **307**, 1915-1920, doi:10.1126/science.1104816 (2005).

9 Hsiao, E. Y. *et al.* Microbiota modulate behavioral and physiological abnormalities associated with neurodevelopmental disorders. *Cell* **155**, 1451-1463, doi:10.1016/j.cell.2013.11.024 (2013).

10 Gill, S. R. *et al.* Metagenomic analysis of the human distal gut microbiome. *Science* **312**, 1355-1359, doi:10.1126/science.1124234 (2006).

11 John H Cummings, G. T. M., and Hans N Englyst. Prebiotic digestion and fermentation. *Am J Clin Nutr* (2001).

12 Laurent Beaugerie, J.-C. P. Antibiotic-associated diarrhoea. *Best Practice & Research Clinical Gastroenterology* **18**, 337-352, doi:10.1016/j.bpg.2003.10.002 10.1053/ybega.2004.439 (2004).

13 Smith, P. M. *et al.* The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* **341**, 569-573, doi:10.1126/science.1241165 (2013).

14 Ramanan, D., Tang, M. S., Bowcutt, R., Loke, P. & Cadwell, K. Bacterial sensor Nod2 prevents inflammation of the small intestine by restricting the expansion of the commensal Bacteroides vulgatus. *Immunity* **41**, 311-324, doi:10.1016/j.immuni.2014.06.015 (2014).

15 Mukherjee, P. K. *et al.* Mycobiota in gastrointestinal diseases. *Nature reviews. Gastroenterology & hepatology*, doi:10.1038/nrgastro.2014.188 (2014).

16 Jesse, R. R. G. W. A. R. U. M. R. Psilocybin can occasion mystical-type experiences having substantial and sustained personal meaning and spiritual significance. *Psychopharmacology* **187:268–283**, doi:10.1007/s00213- (2006).

17 *Center for Disease Control and Prevention. Fungal Diseases. Retrieved*, <http://www.cdc.gov/fungal/diseases/other/exserohilum> (2012).

18 Hooper, L. V. & Macpherson, A. J. Immune adaptations that maintain homeostasis with the intestinal microbiota. *Nature reviews. Immunology* **10**, 159-169, doi:10.1038/nri2710 (2010).

19 Jouault, T. *et al.* Host responses to a versatile commensal: PAMPs and PRRs interplay leading to tolerance or infection by Candida albicans. *Cellular microbiology* **11**, 1007-1015, doi:10.1111/j.1462-5822.2009.01318.x (2009).

20 Underhill, D. & Braun, J. Current understanding of fungal microflora in inflammatory bowel disease pathogenesis. *Inflammatory bowel diseases* **14**, 1147-1153, doi:10.1002/ibd.20402 (2008).

21 Sato, K. *et al.* Dectin-2 is a pattern recognition receptor for fungi that couples with the Fc receptor gamma chain to induce innate immune responses. *The Journal of biological chemistry* **281**, 38854-38866, doi:10.1074/jbc.M606542200 (2006).

22 Round, J. L. & Mazmanian, S. K. The gut microbiota shapes intestinal immune responses during health and disease. *Nature reviews. Immunology* **9**, 313-323, doi:10.1038/nri2515 (2009).

23 Farache, J. *et al.* Luminal bacteria recruit CD103+ dendritic cells into the intestinal epithelium to sample bacterial antigens for presentation. *Immunity* **38**, 581-595, doi:10.1016/j.immuni.2013.01.009 (2013).

24 Loftus, E. V., Jr. & Sandborn, W. J. Epidemiology of inflammatory bowel disease. *Gastroenterology clinics of North America* **31**, 1-20 (2002).

25 Abraham, C. & Cho, J. H. Inflammatory bowel disease. *N Engl J Med* **361**, 2066-2078, doi:10.1056/NEJMra0804647 (2009).

26 Gevers, D. *et al.* The treatment-naive microbiome in new-onset Crohn's disease. *Cell host & microbe* **15**, 382-392, doi:10.1016/j.chom.2014.02.005 (2014).

27 Wu, G. D. *et al.* Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**, 105-108, doi:10.1126/science.1208344 (2011).

28 B. SENDID, J. F. C., 2 P. M. JACQUINOT,3 C. FAILLE,4 J. FRUIT,1 A. CORTOT,2 D. LUCIDARME,2 D. CAMUS,1,3 AND D. POULAIN1,3*. Specific Antibody Response to Oligomannosidic Epitopes in Crohn's Disease. *CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY* **Vol. 3**, p. 219–226 (1996).

29 Standaert-Vitse, A. *et al.* Candida albicans is an immunogen for anti-Saccharomyces cerevisiae antibody markers of Crohn's disease. *Gastroenterology* **130**, 1764-1775, doi:10.1053/j.gastro.2006.02.009 (2006).

30 Standaert-Vitse, A. *et al.* Candida albicans colonization and ASCA in familial Crohn's disease. *The American journal of gastroenterology* **104**, 1745-1753, doi:10.1038/ajg.2009.225 (2009).

31 Ott, S. J. *et al.* Fungi and inflammatory bowel diseases: Alterations of composition and diversity. *Scandinavian journal of gastroenterology* **43**, 831-841, doi:10.1080/00365520801935434 (2008).

32 Ghannoum, M. A. *et al.* Characterization of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS pathogens* **6**, e1000713, doi:10.1371/journal.ppat.1000713 (2010).

33 Wirth, F. & Goldani, L. Z. Epidemiology of Rhodotorula: an emerging pathogen. *Interdisciplinary perspectives on infectious diseases* **2012**, 465717, doi:10.1155/2012/465717 (2012).

34 Heinsbroek, S. E. *et al.* Genetic deletion of dectin-1 does not affect the course of murine experimental colitis. *BMC gastroenterology* **12**, 33, doi:10.1186/1471-230X-12-33 (2012).

35 EDWARD BALISH, M. J. B., CINDY A. SALKOWSKI, KENNETH W. LEE,t AND KENNETH F. BARTIZAL. Colonization of Congenitally Athymic, Gnotobiotic Mice by Candida albicans. *Applied and Environmental Micorbiology*, 647-652 (1984).

36 Sartor, R. B. Mechanisms of disease: pathogenesis of Crohn's disease and ulcerative colitis. *Nature clinical practice. Gastroenterology & hepatology* **3**, 390-407, doi:10.1038/ncpgasthep0528 (2006).

37 Niimura, Y. & Nei, M. Evolution of olfactory receptor genes in the human genome. *Proceedings of the National Academy of Sciences of the United States of America* **100**, 12235-12240, doi:10.1073/pnas.1635157100 (2003).

38 Jung, M. K. *et al.* Erythroid differentiation regulator 1, an interleukin 18-regulated gene, acts as a metastasis suppressor in melanoma. *The Journal of investigative dermatology* **131**, 2096-2104, doi:10.1038/jid.2011.170 (2011).

39 Ivanov, II *et al.* Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell* **139**, 485-498, doi:10.1016/j.cell.2009.09.033 (2009).

40 Salzman, N. H. *et al.* Enteric defensins are essential regulators of intestinal microbial ecology. *Nature immunology* **11**, 76-83, doi:10.1038/ni.1825 (2010).

41 Camilleri, M., Madsen, K., Spiller, R., Greenwood-Van Meerveld, B. & Verne, G. N. Intestinal barrier function in health and gastrointestinal disease. *Neurogastroenterology and motility : the official journal of the European Gastrointestinal Motility Society* **24**, 503-512, doi:10.1111/j.1365-2982.2012.01921.x (2012).

42 Mankertz, J. *et al.* Functional crosstalk between Wnt signaling and Cdx-related transcriptional activation in the regulation of the claudin-2 promoter activity. *Biochemical and Biophysical Research Communications* **314**, 1001-1007, doi:10.1016/j.bbrc.2003.12.185 (2004).

43 Jeremy K. Nicholson, E. H., 1 James Kinross,1 Remy Burcelin,2 & Glenn Gibson, W. J., 4 Sven Pettersson5*. Host-Gut Microbiota Metabolic Interactions. *Science* **336** (2012).

44 Marro, P. J. *et al.* Purine metabolism and inhibition of xanthine oxidase in severely hypoxic neonates going onto extracorporeal membrane oxygenation. *Pediatric research* **41**, 513-520, doi:10.1203/00006450-199704000-00010 (1997).

45 Coutinho Tde, A. *et al.* Associations of serum uric acid with markers of inflammation, metabolic syndrome, and subclinical coronary atherosclerosis. *American journal of hypertension* **20**, 83-89, doi:10.1016/j.amjhyper.2006.06.015 (2007).

46 Musso, G., Gambino, R. & Cassader, M. Interactions between gut microbiota and host metabolism predisposing to obesity and diabetes. *Annual review of medicine* **62**, 361-380, doi:10.1146/annurev-med-012510-175505 (2011).

47 Govani, S. M. & Higgins, P. D. Combination of thiopurines and allopurinol: adverse events and clinical benefit in IBD. *Journal of Crohn's & colitis* **4**, 444-449, doi:10.1016/j.crohns.2010.02.009 (2010).

48 Lioni, M. *et al.* Dysregulation of claudin-7 leads to loss of E-cadherin expression and the increased invasion of esophageal squamous cell carcinoma cells. *The American journal of pathology* **170**, 709-721, doi:10.2353/ajpath.2007.060343 (2007).

49 Turner, J. R. Intestinal mucosal barrier function in health and disease. *Nature reviews. Immunology* **9**, 799-809, doi:10.1038/nri2653 (2009).

50 Martinon, F., Petrilli, V., Mayor, A., Tardivel, A. & Tschopp, J. Gout-associated uric acid crystals activate the NALP3 inflammasome. *Nature* **440**, 237-241, doi:10.1038/nature04516 (2006).

51 RICHET, G. The chemistry of urinary stones around1800: A first in clinical chemistry. *Kidney International* **48**, 876-886 (1995).

52 D. C. HOOPER, S. S., R. B. KEAN, J. M. CHAMPION, G. M. DICKSON, I. CHAUDHRY, AND H. KOPROWSKI. Uric acid, a natural scavenger of peroxynitrite, in experimental allergic encephalomyelitis and multiple sclerosis. *Proc. Natl. Acad. Sci. USA* **Vol. 95,**, 675–680 (1998).

53 BRUCE N. AMES*, R. C., ELIZABETH SCHWIERS*, AND PAUL HOCHSTEINt. Uric acid provides an antioxidant defense in humans against oxidant-andradical-causedagingandcancer:A hypothesis. *Proceedings of the National Academy of Sciences of the United States of America* **78**, 6858-6862 (1981).

54 KelvinJ. A.DAVIES, A., SamarF. MUAKKASSAH-KELLY Paul HOCHSTEIN. Uric acid-iron ion complexes A new aspect of the antioxidantf unctions of uric acid. *Biochemical Journal* **235**, 747-754 (1986).

55 Rock, Y. S. J. E. E. K. L. Molecular identification of a danger signal that alerts the immune system to dying cells. *Nature* **425**, 516-521, doi:10.1038/nature01950 (2003).

56 Bianchi, M. E. DAMPs, PAMPs and alarmins: all we need to know about danger. *Journal of leukocyte biology* **81**, 1-5, doi:10.1189/jlb.0306164 (2007).

57 Meylan, E., Tschopp, J. & Karin, M. Intracellular pattern recognition receptors in the host response. *Nature* **442**, 39-44, doi:10.1038/nature04946 (2006).

58 J. C. EDOZIEN, U. U. U., V. R. YouNG, N. S. SCRIMSHAW. Effects of High Levels of Yeast Feeding on Uric Acid Metabolism of Young Men. *Nature* **228** (1970).

59 Gary D. Buffinton, W. F. D. DEPLETED MUCOSAL ANTIOXIDANT DEFENCES IN INFLAMMATORY BOWEL DISEASE. *ree Radical Biology & Medicine,* **19**, 911-918 (1995).

60 Hooper, D. C. *et al.* Prevention of experimental allergic encephalomyelitis by targeting nitric oxide and peroxynitrite: implications for the treatment of multiple sclerosis. *Proceedings of the National Academy of Sciences of the United States of America* **94**, 2528-2533 (1997).

61 Whiteman, M. & Halliwell, B. Protection against peroxynitrite-dependent tyrosine nitration and alpha 1-antiproteinase inactivation by ascorbic acid. A comparison with other biological antioxidants. *Free radical research* **25**, 275-283 (1996).

62 Edward Balish, R. D. W., Andres Vazquez-Torres, Jessica Jones-Carson, Carey Pierson, and Thomas Warner. Mucosal and systemic candidiasis in IL-8Rh-/- BALB/c mice. *Journal of leukocyte biology* **66** (1999).

63 Boer, W., Folman, L. B., Summerbell, R. C. & Boddy, L. Living in a fungal world: impact of fungi on soil bacterial niche development. *FEMS microbiology reviews* **29**, 795-811, doi:10.1016/j.femsre.2004.11.005 (2005).

64 Wostmann, B. S., Larkin, C., Moriarty, A. & Bruckner-Kardoss, E. Dietary intake, energy metabolism, and excretory losses of adult male germfree Wistar rats. *Laboratory animal science* **33**, 46-50 (1983).

65 Sabri, A. *et al.* Influence of moderate temperatures on myristoyl-CoA metabolism and acyl-CoA thioesterase activity in the psychrophilic antarctic yeast Rhodotorula aurantiaca. *The Journal of biological chemistry* **276**, 12691-12696, doi:10.1074/jbc.M100155200 (2001).

66 Tuohy, K. M. *et al.* Metabolism of Maillard reaction products by the human gut microbiota--implications for health. *Molecular nutrition & food research* **50**, 847-857, doi:10.1002/mnfr.200500126 (2006).